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Correlating Molecular Character of NIR Imaging Agents with Tissue-Specific Uptake

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Abstract

Near-infrared (NIR) fluorescent contrast agents are emerging in optical imaging as sensitive, cost-effective, and nonharmful alternatives to current agents that emit harmful ionizing radiation. Developing spectrally distinct NIR fluorophores to visualize sensitive vital tissues to selectively avoid them during surgical resection of diseased tissue is of great significance. Herein, we report the synthetic variation of pentamethine cyanine fluorophores with modifications of physicochemical properties toward prompting tissue-specific uptake into sensitive tissues (i.e., endocrine glands). Tissue-specific targeting and biodistribution studies revealed localization of contrast agents in the adrenal and pituitary glands, pancreas, and lymph nodes with dependence on molecular characteristics. Incorporation of hydrophobic heterocyclic rings, alkyl groups, and halogens allowed a fine-tuning capability to the hydrophobic character and dipole moment for observing perturbation in biological activity in response to minor structural alterations. These NIR contrast agents have potential for clinical translation for intraoperative imaging in the delineation of delicate glands.

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Supporting Information

Synthetic protocol, characterization, ¹H NMR, ¹³C NMR, and mass spectra. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00475.

Notes

The authors declare no competing financial interest.

INTRODUCTION

Molecular imaging agents that display selectivity toward various tissues are of paramount importance for use in image-guided surgery.^{1–7} For instance, visualizing native endocrine glands during head and neck surgery remains of high clinical significance as current imaging agents being explored utilize the emission of harmful ionizing radiation and place the patient and caregiver at undesirable levels of exposure.^{8,9} Additional disadvantages of common molecular imaging modalities, such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT), include their low spatial resolution of tissue and inability to conduct surgery in real-time with image capture.¹⁰ Optical imaging using near-infrared (NIR) light, however, is harmless as human tissues do not absorb these wavelengths of the electromagnetic spectrum.⁶ Developing fluorophores in the NIR window also helps to minimize the optical background that arises from fluorescence from native biological tissue.^{11–17} This autofluorescence increases background signal which reduces the all-important signal-to-background ratio (SBR) and makes delineating meaningful signal more difficult.

Many groups have utilized a number of approaches for directing molecular imaging agents to particular organs using receptors attached to a reactive moiety residing on the fluorophore.^{18–21} These moieties normally include a carboxylic acid or amine for amide coupling or alkyne/azide for click chemistry which can be synthetically challenging. However, only a few methods have been explored for chemically tailoring a class of fluorophore for site-specific uptake by glands and tissues using physicochemical predictors to systematically synthesize, evaluate, and harness the inherent biodistribution of the fluorophores.²² To date, only one NIR fluorescent compound, indocyanine green (ICG), has been approved for clinical use by the U.S. Food and Drug Administration (FDA).²³ Indeed, the exact molecular structure does not offer appealing biodistribution properties or potential for additional chemical modifications, it serves to validate the cyanine class as an excellent choice for further optimization.²⁴ Cyanine dyes are a unique class of compounds that are generally represented by two nitrogen-containing heterocycles linked via an electron-deficient conjugated polymethine bridge.^{25,26} The absorption and fluorescence properties of these compounds can be tailored, and the two main subclasses of NIR-fluorescing cyanine compounds include pentamethine (~700 nm emission) and heptamethine backbones (~800

nm emission). These compounds have appealing optical properties, including their red-shifted fluorescence, elevated quantum yields, and high stability in serum.

The FLARE (Fluorescence-Assisted Resection and Exploration) imaging system used for intraoperative NIR imaging utilizes two video channels (700 and 800 nm fluorescence) merged with a color video image.^{27,28} We have previously reported a heptamethine cyanine dye (800 nm fluorescence) with unparalleled clearance properties that, after conjugation, selectively images cancerous tissues.^{29,30} For native endocrine-tissue imaging, therefore, we have reserved the lower 700 nm channel for dual imaging of the surgical field. Previously, we elucidated that cyanine chromophores have various binding strengths to serum albumin depending on their hydrophobicity;³¹ therefore, we hypothesized that we could harness this interaction to manipulate the biodistribution of these compounds. Herein, we report the development of pentamethine cyanine imaging agents that display tissue-specific uptake into various endocrine glands resulted from the modification of the heterocyclic nitrogen and methine-bridge.

EXPERIMENTAL DETAILS

Chemicals and Reagents

The chemical reagents used in the synthesis of these compounds were obtained from Acros Organics, Alfa Aesar, and Matrix Scientific. The reactions were followed using silica gel 60 F₂₅₄ thin layer chromatography plates (Merck EMD Millipore, Darmstadt, Germany). Open column chromatography was utilized for the purification of all final compounds using 60–200 μ , 60 Å, classic column silica gel (Dynamic Adsorbents, Norcross, GA). The ¹H NMR and ¹³C NMR spectra were obtained using high quality Kontes NMR tubes (Kimble Chase, Vineland, NJ) rated to 500 MHz and were recorded on a Bruker Avance (400 MHz) spectrometer using DMSO-*d*₆ or MeOD-*d*₄ containing tetramethylsilane (TMS) as an internal calibration standard set to 0.0 ppm. UV–vis/NIR absorption spectra were recorded on a Varian Cary 50 spectrophotometer. High-resolution accurate mass spectra (HRMS) were obtained either at the Georgia State University Mass Spectrometry Facility using a Waters Q-TOF micro (ESI-Q-TOF) mass spectrometer or utilizing a Waters Micromass LCT TOF ES+ Premier mass spectrometer. Liquid chromatography utilized a Waters 2487 single wavelength absorption detector with wavelengths set between 640 and 700 nm depending on the particular photophysical properties. The column used in LC was a Waters Delta-Pak 5 μ M 100 Å 3.9 mm $\times$ 150 mm reversed phase C₁₈ column. Evaporative light scattering detection analyzes trace impurities that cannot be observed by alternate methods; a SEDEX 75 ELSD was utilized in tandem with liquid chromatography to confirm purity (>95% as determined by LC-ELSD-MS).

Synthetic Procedures and Structural Characterizations

This information may be found in Supporting Information.

Optical Property Measurements and Physicochemical Predictions

All optical measurements were performed in various solvents, including ethanol (200 proof, Fischer Chemical), dimethyl sulfoxide (DMSO, 99.9% Acros Organics), phosphate buffered

saline (PBS, pH 7.4, Fischer Scientific), and at 37 °C in 100% fetal bovine serum (FBS, Fischer Scientific) buffered with 50 mM HEPES, pH 7.4. Absorbance and fluorescence emission spectra of the series of NIR fluorophores were measured using Varian Cary 50 absorbance spectrophotometer (190–1100 nm) and Shimadzu RF-5301PC spectrofluorometer (350–1000 nm). For fluorescence quantum yield (QY) measurements, rhodamine 800 in absolute ethanol (QY = 28%) was used as a calibration standard under conditions of matched absorbance at 640 nm. In silico calculations of physicochemical distribution coefficient (log *D* at pH 7.4) was calculated using Marvin and JChem calculator plugins (ChemAxon, Budapest, Hungary). Electrostatic maps were calculated using Spartan DFT calculations at the B3LYP level.

LC-MS Spectroscopy

The purity of all compounds was measured using liquid chromatography–mass spectrometry (LC-MS) on a Waters system consisting of a 1525 binary HPLC pump with a manual 7725i Rheodyne injector, a 996 photodiode array (PDA) detector, and a 2475 multiwavelength fluorescence detector. The column eluent was divided in 2 using a flow splitter (Upchurch Scientific). A portion of the eluent flowed into an ELSD (Richards Scientific), while the rest flowed into a Micromass LCT ESI-TOF spectrometer (Waters) equipped with a Symmetry (R) C18 (4.6 mm × 150 mm, 5 μm) reverse-phase HPLC column. For mass spectrometry, mobile phase was solvent A = 0.1% formic acid (FA) in water and solvent B = CH₃CN with 95% A for 5 min and a linear gradient from 5% to 40% CH₃CN (from A to B for 30 min) at a flow rate of 1 mL/min, capillary voltage was –3317 V, and sample cone voltage was –50 V.

Optical Imaging System and Fluorescence Assisted Surgical Resection

The real-time intraoperative FLARE imaging system has been described in detail previously.^{27,28} In this study, 670 nm excitation was used at a fluence rate of 4 mW/cm², with white light (400– 650 nm) at 40000 lx. Color and NIR fluorescence images were acquired simultaneously with custom software at rates up to 15 Hz over a 15 cm diameter field of view. The imaging system was positioned at a distance of 18 in. from the surgical field. The wavelength used for excitation and emission filters was 650 ± 22 nm.

Animal Models

Animals were housed in an AAALAC-certified facility and were studied under the supervision of Beth Israel Deaconess Medical Center's Institutional Animal Care and Use Committee (IACUC) in accordance with approved institutional protocols (no. 101-2011 for rodents and no. 034-2013 for pigs). Male CD-1 mice (20–30 g, 5–6 weeks, Charles River Laboratories, Wilmington, MA) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally (Webster Veterinary, Fort Devens, MA). Female Yorkshire pigs (E.M. Parsons and Sons, Hadley, MA) averaging 35 kg were induced with 4.4 mg/kg intramuscular Telazol (Fort Dodge Laboratories, Fort Dodge, IA), intubated, and maintained with 2% isoflurane (Baxter Healthcare Corp., Deerfield, IL). Following anesthesia, electrocardiogram, heart rate, pulse oximetry, and body temperature were monitored throughout surgery.

Quantitative Analysis

At each time point, the fluorescence and background intensity of a region of interest (ROI) over each tissue was quantified using custom FLARE software. The signal-to-background ratio (SBR) was calculated as $SBR = \text{fluorescence}/\text{background}$, where background is the signal intensity of neighboring tissues, such as muscle or skin, obtained over the imaging period. The SBR of each organ/tissue relative to the abdominal wall was quantified and labeled as: -, 1-2; +, 2-3; ++, 3-5; and +++, >5. All NIR fluorescence images for a particular fluorophore were normalized identically for all conditions of an experiment. At least five animals were analyzed. Statistical analysis was carried out using the unpaired Student's t-test or one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Synthesis of Pentamethine Fluorophores

Using our optimized synthetic strategy for preparing pentamethine cyanines that exhibit varied hydrophobic character, we began by reacting either mucochloric 1 or mucobromic 2 acid with aniline in warm ethanol and upon cooling and dilution with diethyl ether, the intermediate salts 4 and 5 precipitated as yellow solids, with precursor 3 being commercially obtained. In parallel, heterocyclic derivatives 8-15 were prepared from 2,3,3-trimethylindolenine 6 and 1,1,2-trimethyl-1*H*-benzo[e]-indole 7 using the corresponding alkyl halide in boiling acetonitrile. Subsequently, the reaction of individual salts 8-15 with intermediate 3-5 formed the final pentamethine cyanines that were purified by open column chromatography or via our optimized precipitation procedure from methanol using dilution with either diethyl ether or methyl *tert*-butyl ether until analytical purity (>95% as determined by LC-ELSD-MS) was obtained for evaluating these compounds in biodistribution experiments. Note: Cellular experiments were conducted regarding the following compounds and are previously referred to using ESNF nomenclature: 16 (ESNF-10), 22 (ESNF-11), 25 (ESNF-12), and 31 (ESNF-14).³² Herein, we will discuss the animal studies concerning the entire systematic set of fluorophores and will be referred to by their numbers in synthetic Scheme 1.

Optical and Physicochemical Properties

The optical properties, shown in Table 1, including λ_{max} (ethanol and FBS), λ_{em} (ethanol and FBS), extinction coefficient (ethanol, DMSO, PBS, and FBS), and fluorescence quantum yield (FBS) of the synthesized fluorophores, were determined to ensure compatibility and visibility with the FLARE imaging system. All compounds showed excellent extinction coefficients in the near-infrared region in all four of the studied solvents, with many compounds having satisfactory optical properties in serum for effective detection *in vivo*. The more hydrophobic fluorophores displayed a second blue-shifted absorption maximum due to H-aggregation in the highly polar FBS (Figure 1). Fortunately, we did not encounter loss in signal resulting from fluorescence quenching from microaggregation *in vivo*. The quantum yield values are very high in serum, suggesting great applicability in NIR-fluorescence imaging. To correlate *in vivo* biodistribution to the molecular characteristics of the synthesized fluorophores, the physicochemical properties (Log *D*, molecular weight and rotatable bonds) were predicted using ChemAxon (JChem plugin) with molecular dipole

moment and volume being calculated using Spartan 10 (Wave Function, Inc., Irvine, CA). The molecular weight values are independent of counterion as the fluorophore and anion would become disassociated in vivo. The molecular weight values fall between 383.55 and 770.86, and the distribution coefficient numbers fall between 3.56 and 10.85, with the halogenation at the central carbon, heterocyclic variation, and alkyl length alteration providing fine-tuning characteristics to the hydrophobicity of the compounds for observing minor perturbation to the biological profile. Similarly, the molecular volume for the synthesized compounds falls between 445.39 and 806.74 and the compounds exhibit 0, 4, or 8 rotatable bonds.

Structure-Inherent Targeting Ability of Hydrophobic NIR Fluorophores

To assess the in vivo performance of the newly prepared pentamethine cyanines, we injected them into CD-1 mice and observed the passive localization and overall biodistribution. The synthesized compounds displayed very appealing structure-inherent endocrine targeting of biological tissues for intraoperative guidance using NIR light. Surgeons require precise visualization of sensitive tissues to avoid accidental transection, and we have focused our biodistribution study on the thyroid glands, adrenal glands, pancreas, and lymph nodes.

To draw conclusions from these results and optimize a model for predicting the in vivo performance of NIR fluorophores, we attempted to correlate calculated physicochemical predictors with the biodistribution and localization within the targeted tissues. We have noticed a general trend for all of the tissues where the overall hydrophobicity (Log *D*, pH 7.4) greatly controls the in vivo fate of the molecules. Specifically, thyroid gland (TG) targeting seems to be effective within a range of Log *D* values. Observing the trend in Table 2, we see that the optimum hydrophobicity falls within 3.56 to 6.49. Exemplifying this, we compared the butylated set of indolenine compounds 22–24 that spans the upper range we have set for TG targeting. The compound with a *meso*-hydrogen atom 22 localizes effectively in the thyroid gland as seen in Figure 2. The addition of *meso*-halogens 23–24 to the butylated indolenine set of compounds drastically lowers the thyroid targeting ability in deference to nonspecific uptake in the lung resulting from an increase in hydrophobicity, which also may lead to higher enveloping by macrophages which carry the fluorophore to the liver for destruction and filtration.

Similarly, we expected that because the TG and adrenal gland (AG) are both integrated into the endocrine system, they may be effectively targeted using similar molecular character. When comparing the adrenal gland uptake of compounds 34–36, all containing a benzo[*e*]indolenine heterocyclic scaffold with a highly hydrophobic butyl group, we notice that the addition of *meso* halogens greatly reduces the successful targeting abilities (Figure 3). Further examining the AG targeting set, we see that a wide range of Log *D* values spanning the entire set of compounds (3.56–10.85) are tolerated; however, *meso*-halogenation universally reduces the AG retention as seen in Table 2.

The methylated dyes, 16–18, showed an interesting trend in pancreatic tissue (Pa) targeting (Figure 4). The non-halogenated 16 (Log *D* = 3.56) displays increased signal in the pancreas compared to background signal, with most of the fluorophore molecules being cleared through renal filtration; correspondingly, the halogenated compounds, with slightly

increased hydrophobicity (Log D = 3.79 and 3.96, respectively) and lower overall dipole moment shown in the electrostatic maps, shows slightly decreased SBR in the pancreas compared to 16. Observing the entire set of synthesized fluorophores, we observe that the lower Log D compounds with smaller molecular volume and smaller molar mass exhibit higher pancreatic uptake with compound 16 showing excellent localization.

The last tissues that we aimed to target were the lymph nodes (LN), which are critical in locating during surgery to either remove, biopsy, or avoid. We see that as a general trend, the compounds exhibiting higher hydrophobicity localize within the LN with higher SBR compared to more hydrophilic fluorophores; however, closely observing the correlation between LN targeting and Log D reveals that LN localization cannot be solely attributed to the hydrophobic character, although we have observed that the general hydrophobic nature of a compound greatly controls the in vivo fate in previous glands. Building on this principle, we have noticed that the localization within the lymph node is dependent on hydrophobicity, with overall dipole moment acting as a fine-tuning characteristic. It has been previously reported that altering the dipole moment of steroid-based compounds drastically alters the overall biodistribution pattern,³³ and we have noticed a similar trend when comparing compounds 28–33 (Figure 5). Compounds bearing a *meso*-hydrogen atom 28 and 31 display very high dipole moments ($\mu > 3.80$ debye); however, the *meso*-halogenated compounds display comparatively low dipole moments ($\mu < 1.85$ debye) due to the inductive electron withdrawing characteristics acting adversely to the overall dipole. Of all the physicochemical descriptors, only net dipole offers an effective explanation for the observation that only *meso*-halogen-bearing compounds localize effectively within the lymph node versus the hydrogen-containing parent compound within the effective Log D range.

CONCLUSIONS

As the clinical need for intraoperative NIR contrast agents reaches a critical level, we have successfully generated a series of pentamethine cyanine fluorophores for the targeting of vital tissues that should be carefully avoided during surgery. The targetability of these compounds represents the first successful correlation of tissue-specific contrast agents with their physicochemical properties reported to date that could help solve an unmet clinical need in image-guided surgery. We are currently investigating the cellular targets that explain this interesting phenomenon which will be thoroughly explored in subsequent publications. Additionally, it should be noted that the physiological filtration and clearance pathways of these compounds are highly consistent with the Lipinski's rules, with the more hydrophobic compounds being observed in the liver and the less hydrophobic contrast agents being cleared through renal filtration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Absorption and emission spectra for each of the evaluated compounds (0.3 μ M) in 100% FBS supplemented with 50 mM HEPES buffer at 37 $^{\circ}$ C.

Thyroid Gland Targeting NIR Fluorophores

Figure 2.

Thyroid gland (TG)-targeted NIR fluorophores: 10 nmol of each fluorophore was injected intravenously into CD-1 mice 4 h prior to imaging and resection. Abbreviations used are: Du, duodenum; He, heart; In, intestine; Ki, kidney; Li, liver; Lu, lung; Mu, muscle; Pa, pancreas; SG, salivary gland, Sp, spleen. Arrows indicate TGs. Scale bars = 1 cm.

Figure 3.

Adrenal gland (AG)-targeted NIR fluorophores: 10 nmol of each fluorophore was injected intravenously into CD-1 mice 4 h prior to imaging and resection. Abbreviations used are: Du, duodenum; He, heart; In, intestine; Ki, kidney; Li, liver; LN, lymph nodes; Lu, lung; Mu, muscle; Pa, pancreas; Sp, spleen. Arrows indicate AGs. Scale bars = 1 cm.

Pancreas Targeting NIR Fluorophores

Figure 4.

Pancreas (Pa)-targeted NIR fluorophores: 10 nmol of each fluorophore was injected intravenously into CD-1 mice 4 h prior to imaging and resection. Abbreviations used are Du, duodenum; He, heart; In, intestine; Ki, kidney; Li, liver; Lu, lung; Mu, muscle; Pa, pancreas; Sp, spleen. Arrows indicate Pa. Scale bars = 1 cm.

Figure 5.

Lymph node (LN)-targeted fluorophores. (A) Methylated benz[e]indolenine-based cyanines and (B) ethylated benz[e]indolenine-based cyanines: 10 nmol of each fluorophore was injected intravenously into CD-1 mice 4 h prior to imaging and resection. Abbreviations used are: Du, duodenum; He, heart; In, intestine; Ki, kidney; Li, liver; Lu, lung; Mu, muscle; Pa, pancreas; Sp, spleen. Arrows indicate LNs. Scale bars = 1 cm.

Scheme 1.

Synthetic Route to the Final Hydrophobic Pentamethine Fluorophores

Table 1

Optical Properties of NIR Fluorophores^a

ID	λ_{abs} (nm)	EtOH/FBS	λ_{em} (nm)	EtOH/FBS	SS (nm)	EtOH/FBS	extinction coefficient ($\text{M}^{-1} \text{cm}^{-1}$)				QY (%) FBS
							EtOH	DMSO	PBS	FBS	
16	645/649		660/662		15/13		212500	248200	218800	184500	31.0
17	644/638		659/665		15/27		235300	207800	171000	168800	28.8
18	641/634		657/660		16/26		257330	253200	204400	211700	43.6
19	645/637		667/665		22/28		246500	235000	179800	193300	17.0
20	646/649		662/659		16/10		262200	237000	213600	204000	25.5
21	642/636		655/656		13/20		211400	210000	176800	147300	43.7
22	644/640		666/668		22/28		210200	182800	177600	140200	16.0
23	649/644		665/661		16/17		231400	143600	80600	123900	14.8
24	646/640		661/660		15/20		218300	134600	73600	135200	15.7
25	655/646		669/666		14/20		229700	178000	56600	139200	17.7
26	652/653		664/665		12/12		254500	228200	62000	113000	22.1
27	650/648		665/660		15/12		241800	211200	61200	117000	28.6
28	680/688		701/711		21/23		177500	167600	60000	168400	17.5
29	681/689		697/702		16/13		218200	206400	77200	214900	18.0
30	676/682		699/699		23/17		164800	173400	54600	161100	17.9
31	682/691		702/705		20/14		233400	203200	97600	202400	13.8
32	684/690		700/705		16/15		215200	172800	86000	207400	21.9
33	681/682		695/698		14/17		182000	202600	68200	177200	11.8
34	684/691		706/711		22/20		255500	237000	76200	193300	8.9
35	685/691		703/702		18/11		233800	201200	56600	157100	12.0
36	683/689		702/699		19/10		222800	212600	62800	170300	22.7
37	685/698		711/710		26/12		194200	95800	39400	166700	14.5
38	687/697		705/707		18/10		164000	158600	42600	143400	17.9
39	685/695		704/709		19/14		285200	220200	56400	133900	12.9

^a All measurements were performed in several solvents, ethanol (EtOH), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), and fetal bovine serum (FBS), pH = 7.4 at 37 °C, and the fluorescence QY in FBS, pH = 7.4 at 37 °C. Abbreviations: λ_{abs} , peak absorbance; λ_{em} , peak emission at 640 nm excitation; SS, Stokes shift; QY, quantum yield.

Table 2

Targetability and Biodistribution of NIR Fluorophores for Endocrine Glands at 4 h Postinjection^a

ID	Log D (pH 7.4)	volume (Å ³)	rotatable bonds	dipole (debye)	MW (g/mol)	targeted tissues					biodistribution				
						TG	AG	Pa	LN	Li	Ki	Lu	Sp	SG	
16	3.56	445.39	0	3.26	383.55	++	+++	+++	+	-	+++	+	-	++	
17	3.79	458.97	0	1.88	417.99	++	+	++	+	+	+++	++	-	+++	
18	3.96	462.36	0	1.50	462.45	++	+	+	+	+	+++	++	-	++	
19	4.27	482.09	4	2.10	411.60	+++	+	++	+	+	+++	++	-	+++	
20	4.51	495.44	4	0.32	446.05	++	-	++	+	++	+++	++	-	+++	
21	4.67	499.85	4	1.35	490.5	+	-	++	+	+++	+++	+++	-	+++	
22	6.20	573.88	8	2.12	467.71	+++	+++	++	+	++	+++	++	+	++	
23	6.44	587.27	8	3.47	502.15	++	++	++	+++	+++	++	+++	++	++	
24	6.61	591.69	8	3.44	546.60	-	++	++	+++	+++	++	+++	++	++	
25	8.47	686.97	8	3.42	591.85	-	++	+	++	++	++	+++	++	+	
26	8.71	700.36	8	5.05	626.29	-	+	+	++	+++	++	+++	+++	+	
27	8.87	704.78	8	5.43	670.74	-	+	+	+	+++	++	+++	+++	+	
28	5.54	546.41	0	4.45	483.67	++	+++	+	+	+++	++	+++	++	+	
29	5.77	559.86	0	1.83	518.11	++	+	+	+++	+++	++	+++	++	+	
30	5.94	564.29	0	0.67	562.56	++	+	+	+++	+++	++	+++	+++	+	
31	6.25	583.80	4	3.84	511.72	++	+++	+	+	+++	++	+++	++	++	
32	6.49	597.23	4	1.36	546.16	++	++	+	+++	++	++	++	++	++	
33	6.65	601.67	4	0.30	590.62	+	++	+	+++	+++	++	++	++	++	
34	8.18	657.21	8	0.71	567.83	+	+++	+	++	+++	++	+++	++	++	
35	8.42	670.60	8	1.04	602.27	+	++	+	++	+++	++	+++	+++	++	
36	8.59	675.02	8	1.04	646.72	+	-	+	++	+++	++	+++	+++	+	
37	10.45	787.63	8	2.88	691.96	-	+++	+	+++	++	-	+	+	+	
38	10.69	800.94	8	4.42	726.41	+	+	+	+++	+++	++	+++	+++	+	
39	10.85	806.74	8	5.82	770.86	+	+	+	+++	+++	++	+++	+++	++	

^a Abbreviations used are: TG, thyroid gland; AG, adrenal gland; Pa, pancreas; LN, lymph node; Li, liver; Ki, kidneys; Lu, lungs; Sp, spleen; SG, salivary gland. The SBR of each organ/tissue relative to the abdominal wall was quantified and labeled as -, 1-2; +, 2-3; ++, 3-5; +++, >5.